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REVIEW

VIP and PACAP: neuropeptide modulators of CNS inflammation, injury, and repair

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Inflammatory processes play both regenerative and destructive roles in multiple sclerosis, stroke, CNS trauma, amyotrophic lateral sclerosis and aging-related neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's. Endogenous defence mechanisms against these pathologies include those that are directly neuroprotective, and those that modulate the expression of inflammatory mediators in microglia, astrocytes, and invading inflammatory cells. While a number of mechanisms and molecules have been identified that can directly promote neuronal survival, less is known about how the brain protects itself from harmful inflammation, and further, how it co-opts the healing function of the immune system to promote CNS repair. The two closely related neuroprotective peptides, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase-activating peptide (PACAP), which are up-regulated in neurons and immune cells after injury and/or inflammation, are known to protect neurons, but also exert powerful *in vivo* immunomodulatory actions, which are primarily anti-inflammatory. These peptide actions are mediated by high-affinity receptors expressed not only on neurons, but also astrocytes, microglia and peripheral inflammatory cells. Well-established immunomodulatory actions of these peptides are to inhibit macrophage and microglia production and release of inflammatory mediators such as TNF-α and IFN-γ, and polarization of T-cell responses away from Th1 and Th17, and towards a Th2 phenotype. More recent studies have revealed that these peptides can also promote the production of both natural and inducible subsets of regulatory T-cells. The neuroprotective and immunomodulatory actions of VIP and PACAP suggest that receptors for these peptides may be therapeutic targets for neurodegenerative and neuroinflammatory diseases and other forms of CNS injury.

Abbreviations

APE1, apurinic/apyrimidinic endonuclease; IL-1Ra, IL-1 receptor antagonist; IML, intermediolateral; KO, knockout; MOG_{35-55}, myelin oligodendrocyte glycoprotein, amino acids 35-55; MS, multiple sclerosis; MΦ, macrophage; NA, noradrenaline; PACAP, pituitary adenylyl cyclase-activating peptide; PGC1α, peroxisome proliferator-activated receptor γ co-activator 1α; SCG, superior cervical ganglia; SNS, sympathetic nervous system; Treg, regulatory T-cell; VIP, vasoactive intestinal peptide

Introduction

The brain was once thought to be excluded from immune surveillance and other inflammatory processes except under a few specific pathological conditions such as CNS infection and multiple sclerosis (MS). Indeed foreign antigens are quite limited in their ability to elicit robust T-cell or humoral responses in the CNS due to a relative lack of lymphoid drainage and limited expression of major histocompatibility complex molecules. Moreover, neither strong innate nor amplified adaptive inflammatory responses are generally observed in the brain due to the presence of tight endothelial junctions and astrocyte endfeet that provide a barrier against influx of inflammatory cells. A long-standing view in neuroscience that the brain is a highly immunoprivileged organ, as opposed to one that is relatively immune-privileged, however, impeded progress in our understanding of fundamental mechanisms by which the brain attempts to protect

and repair itself in common pathologies Fortunately, this view is changing with newly identified genetic linkages of immune-related genes with neurological diseases and the realization that perhaps all neurodegenerative diseases and even certain mental health disorders such as autism are often accompanied by neuroinflammation and peripheral markers of inflammation. Attesting to this change of thinking was the dedication in 2009 of an entire issue of *Neuron* to neural-immune interactions, and issues of *Nature Reviews Immunology* and *Nature Neuroscience* in 2009 and 2012, respectively, with a major focus on the topic of neuroimmunology. The following review is intended to describe how two neuropeptides, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase-activating peptide (PACAP), may act in neurological diseases as neuroprotective and immunomodulatory factors.

Significance of inflammation in the nervous system

It has been presumed that the process of neurotransmission in the brain might be too delicate to withstand the robust release of reactive oxygen species during active inflammation. While this may be true, an estimated 10–20% of brain cells are microglia, the resident macrophages (MΦ) of the CNS. These cells, along with astrocytes, can be activated under various pathological conditions to produce a host of molecules, some of which are proinflammatory, while others are anti-inflammatory, neuroprotective or regenerative. In addition, it is now clear that perivascular MΦ are abundantly associated with the leptomeningeal blood vessels on the surface of the brain and major penetrating blood vessels. Moreover, in normal animals, T lymphocytes constantly flux in and out of the brain parenchyma. Whether or not these T-cells function to scan for foreign or altered antigens or if they are involved in tolerance or protection remains to be firmly established. In any case, the presence of myeloid and lymphocytic cells within or near the brain parenchyma implies that at least some degree of immune cell activity may occur in the CNS, most likely to deter infection, provide tolerance and/or to abet healing after injury. Finally, it must be considered that the normal physical and molecular barriers to immune cell flux can be severely disrupted in stroke, CNS trauma and autoimmune diseases such as MS, and in some cases are overwhelmed by infectious agents. It thus seems likely that additional mechanisms need to be deployable by the nervous system to modulate excessive CNS inflammation, prevent damage and promote healing after stroke, trauma and perhaps neurodegeneration. As discussed below, neuropeptides such as VIP and PACAP are strongly up-regulated in injury and inflammation and function in these capacities.

VIP, PACAP, ligands and receptors: general biological functions and signalling mechanisms

VIP was discovered in 1970 as a 28-amino acid polypeptide in intestinal extracts capable of inducing system vasodilation, and later found to be present in myenteric and submucosal gastrointestinal neurons, but also in specific populations of neurons of the central, autonomic and sensory nervous systems. PACAP was discovered almost two decades later as a 38-amino acid hypothalamic neuropeptide (and a carboxy-terminal truncated 27-amino acid form) 70% identical to VIP that potently induced cAMP levels in pituitary cells (Miyata *et al.*, 1989). Although widely regarded as neuropeptides that meditate or modulate diverse processes in the mammalian brain such as circadian rhythms and stress responses (reviewed in Vaudry *et al.*, 2009), considerable evidence implicate PACAP and VIP as neuroprotective and regeneration factors in the diseased and injured brain (reviewed in Brenneman, 2007; Dejda *et al.*, 2008; Reglodi *et al.*, 2011; Seaborn *et al.*, 2011). Moreover, systemic administration of these peptides is reported by several groups to be efficacious in animal models of CNS pathologies such as stroke (Chen *et al.*, 2006; Ohtaki *et al.*, 2006; Stetler *et al.*, 2010), MS (Kato *et al.*, 2004; Gonzalez-Rey *et al.*, 2006) and Parkinson's disease (Reglodi *et al.*, 2004; Wang *et al.*, 2008).

Three heterotrimeric GPCRs mediate the actions of VIP and PACAP, officially named by IUPHAR as PAC_1, $VPAC_1$ and $VPAC_2$ (Harmar *et al.*, 1998), each with a unique expression pattern. Of these, PAC_1 binds only PACAP with high affinity, whereas $VPAC_1$ and $VPAC_2$ can bind avidly either VIP or PACAP (Harmar *et al.*, 2012; Figure 1). The genes encoding PACAP and VIP were recently given the gene names *ADCYAP1* and *VIP*, respectively, and the receptors

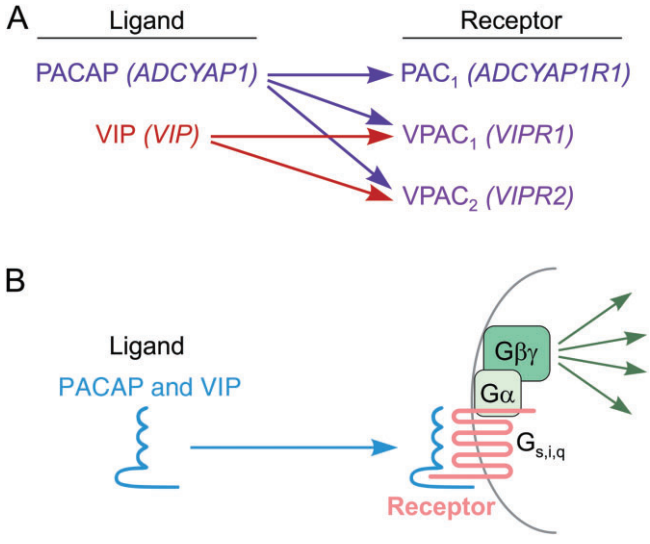

Figure 1

VIP/PACAP ligand/receptor interactions. (A) IUPHAR and gene names are indicated in standard text and italics respectively. PAC_1 receptors are highly selective for PACAP and are generally only responsive to PACAP. $VPAC_1$ and $VPAC_2$, on the other hand, serve as physiological receptors for either PACAP or VIP, depending on the specific neuropeptide released from nearby cells or axon terminals. (B) General interaction of PACAP and VIP with their cell surface receptors. Each of the receptors is a seven-transmembrane GPCR coupled primarily via Gs to adenylate cyclase. Other pathways can be activated via βγ subunits, and by alternative coupling of the receptors to other G-proteins such as Gi and Gq (see text).

ADCYAP1R1, *VIPR1*, *VIPR2*. Unfortunately, the receptor gene nomenclature may be incorrectly interpreted to imply that the receptors encoding by *VIPR1* and *VIPR2* function mainly as VIP receptors. This may explain why recent papers reporting the linkage of *VIPR2* to schizophrenia (Levinson *et al.*, 2011; Vacic *et al.*, 2011) discussed only VIP as the relevant ligand, giving no mention to the fact that the receptor encoded by *VIPR2* binds PACAP with an affinity equal to or higher than VIP. It is thus important to recognize that *VIPR1* and *VIPR2* encode physiological receptors for either VIP or PACAP, depending on the specific neuropeptide released from nearby cells or axon terminals.

Each of these receptors is coupled primarily to Gs, and activates adenylyl cyclase and PKA, but other pathways are often activated or inhibited in some cells in parallel or downstream of cAMP, including pathways involving exchange proteins activated by cAMP (Ster *et al.*, 2007), NO (Murthy *et al.*, 1993), PLC (Spengler *et al.*, 1993), phosphatidylinositol 3-kinase (Straub and Sharp, 1996), src (Koh, 1991), MAPK (Barrie *et al.*, 1997; Villalba *et al.*, 1997; Lelievre *et al.*, 1998), Jak/STAT and NF-κB (Delgado and Ganea, 1999; 2000).

Up-regulation of the VIP/PACAP ligand/receptor signalling system in neurons after injury and during inflammation

Considerable effort has been made to understand how the VIP/PACAP ligand/receptor signalling system responds in various injury models and the mechanisms involved. A number of laboratories have utilized peripheral nerve injury models, which have the advantage that they can be carefully controlled and mechanistically dissected. Those studies have repeatedly demonstrated strong up-regulation in PACAP and/or VIP in injured neurons, including motor (Zhou *et al.*, 1999), sympathetic (Mohney *et al.*, 1994; Moller *et al.*, 1997) and sensory neurons (Zhang *et al.*, 1995). PACAP gene expression was also shown to be up-regulated in sensory neurons following target inflammation (Zhang *et al.*, 1998) and both VIP and PACAP mRNA were induced in motor neurons following application of an inflammatory stimulus directly to the nerve (Armstrong *et al.*, 2004). Mechanisms responsible for the up-regulation of VIP and PACAP in sympathetic neurons have been investigated in detail (reviewed in Zigmond, 2011), and found to involve both loss of target-derived factors and an induction in expression of cytokines in the gp130 family (such as leukaemia inhibitory factor), a pathway shown by others to regulate VIP gene transcription via STAT proteins and cytokine-responsive elements on the VIP gene (Symes *et al.*, 1995; Jones *et al.*, 2000). Using a different approach, it was shown that the induction of PACAP gene expression in facial motor neurons after axotomy was blocked in SCID mice (which lack lymphocytes), but maintained in SCID mice pre-infused with wild-type (WT) splenocytes (Armstrong *et al.*, 2003), providing evidence that inflammatory cells *per se* are required in this injury model for the induction of neuropeptide expression after injury.

Other investigations have examined changes in PACAP and PAC_1 receptor expression in cortical and hippocampal neurons after CNS injury. For example, PACAP gene transcripts were found to be significantly induced in pyramidal neurons in layers II-III of the cortex after focal ischaemic injury (Stumm *et al.*, 2007). Other studies indicated that gene expression for PAC_1 receptors was increased and $VPAC_2$ receptors decreased in the mouse hippocampus in response to ischaemia induced by bilateral common carotid artery occlusion (Nakamachi *et al.*, 2012b). In a model of moderate traumatic injury to rat brain cortex, PACAP gene expression was found to be induced in the ipsilateral cortex, especially in the perifocal region, and also within the dentate gyrus of the hippocampus (Skoglosa *et al.*, 1999). In a model of cortical focal cold injury, $VPAC_2$ receptor immunoreactivity was up-regulated in reactive astrocytes, whereas VIP became expressed in microglia (Nishimoto *et al.*, 2011). Finally, gene expression for both PACAP and PAC_1 were induced in the spinal cord after moderate compression injury (Tsuchikawa *et al.*, 2012). Immunohistochemistry colocalization studies demonstrated that PACAP was induced in motor neurons, whereas PAC_1 was induced in both motor neurons and astrocytes. The changes in expression of these ligands and receptors after different forms of injury suggest that PACAP and VIP signalling systems might be involved in attempts to minimize the impact of injury and/or to promote recovery. Proposed actions of these peptides and potential mechanisms involved are described in the following sections.

Direct actions of VIP and PACAP on neuronal survival, axon integrity and axonal growth

VIP and PACAP are pleiotropic growth factors, affecting proliferation, differentiation, survival and maturation of multiple neural and non-neural cell types. These actions have been extensively reviewed elsewhere (Waschek, 1995; Waschek *et al.*, 2000; Waschek, 2002; Brenneman, 2007; Falluel-Morel *et al.*, 2007; Dejda *et al.*, 2008; Nakamachi *et al.*, 2011; Reglodi *et al.*, 2011; Seaborn *et al.*, 2011; Shioda and Gozes, 2011; Nakamachi *et al.*, 2012a) so the discussion here will be limited to points considered salient for this review and new findings that suggest novel mechanisms of protection. Perhaps the most well-studied and best understood growth factor-like action of these peptides is the PACAP inhibition of neuronal apoptosis. In this regard, PACAP has been reported to promote the survival of numerous neuronal cell types in culture, including cortical, dopaminergic, motor, olfactory and cerebellar granule neurons, neural stem/progenitors and PC12 pheochromocytoma cells (reviewed in Waschek, 2002; Dejda *et al.*, 2008; Seaborn *et al.*, 2011). Most mechanistic work has been performed on cerebellar granule neurons and supports a model whereby PACAP acts in these cells on PAC_1 receptors to trigger both non-canonical and canonical cAMP/PKA pathways to induce of Bcl-2 gene expression (Dejda *et al.*, 2008; Falluel-Morel *et al.*, 2008). PAC_1 signalling in these cells is also reported to trigger a more rapid, but as yet undefined pathway that results in inhibition of caspase 3 activity but does not require intervening protein synthesis (Falluel-Morel *et al.*, 2004).

Loss of axons and/or dendrites independent of (or at least preceding) neuron loss appears to be involved in most neurodegenerative diseases and certain neuropsychiatric afflictions such as schizophrenia. Thus, a potentially important goal is to identify mechanisms normally employed by the brain to minimize axonal and dendritic loss. Furthermore, regeneration of axons occurs concurrently with axon degeneration in MS and other neurodegenerative diseases, suggesting that mechanisms that promote axonal regeneration can also be targeted in these pathologies. In these regards, other well-described, albeit less-understood actions of PACAP and VIP include their abilities to promote the integrity and growth of axons (reviewed in Waschek, 2002; Nakamachi *et al.*, 2011). We addressed the *in vivo* relevance of PACAP action on axonal growth a few years ago, demonstrating that PACAP-deficient (knockout, KO) mice exhibited delayed axonal regeneration in a facial nerve crush model (Armstrong *et al.*, 2008), while others have provided evidence that PACAP administration *in vivo* can attenuate axon degeneration in CNS injury models (Chen and Tzeng, 2005; Tamas *et al.*, 2006). *In vitro* studies implicate PAC_1 receptors, PKA and MAPK as mediating the effects of PACAP to enhance neurite outgrowth (Lu and DiCicco-Bloom, 1997; Guirland *et al.*, 2003; Monaghan *et al.*, 2008; Emery and Eiden, 2012), although downstream effectors remain relatively ill defined. Very recently, using primary hippocampal neurons cultures, it was reported that PACAP promoted neuritogenesis in association with enhanced mitochondria membrane potential, and increased the expression of peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), a master transcriptional co-regulator of mitochondria biogenesis and activation (Kambe and Miyata, 2012). While PGC1α activity is posttranslationally induced during high-energy demand (high AMP/ATP ratio) via the AMP-activated protein kinase, a lesser known mode of PGC1α regulation occurs at the level of gene expression. This operates in the context of plasticity and/or following external cellular signals such as β-adrenergic stimulation, and apparently mediated by cAMP-responsive element-binding protein (Puigserver *et al.*, 1998; Handschin *et al.*, 2003; Puigserver and Spiegelman, 2003; Cui *et al.*, 2006; St-Pierre *et al.*, 2006; Cheng *et al.*, 2010; Hondares *et al.*, 2011). Thus, PACAP-mediated induction of mitochondria biosynthesis might allow increased ATP synthesis during axonal growth. Alternatively, a PGC1α-mediated PACAP up-regulation of mitochondria biogenesis might also serve to maintain axon integrity during inflammation and/or oxidative stress by replacing mitochondria that have been damaged and removed by mitophagy.

Another potentially relevant finding linking PACAP to mitochondrial biology came from a study in which intracerebroventricular PACAP administration inhibited oxidative DNA stress and promoted survival in CA1 hippocampal neurons in a rat global ischaemia model (Stetler *et al.*, 2010). The pertinent finding was that this occurred in association with induced expression of apurinic/apyrimidinic endonuclease (APE1), a DNA repair enzyme that localizes to mitochondria in response to oxidative stress. APE1 knockdown *in vivo* abrogated the neuroprotective action of PACAP, a finding confirmed *in vitro* on cultured neurons. Thus, a hitherto unrecognized core function of PACAP/PAC_1 signalling in stressed neurons might be to maintain a healthy pool of mitochondria within axons and neural cell bodies, thereby providing mechanisms to protect neurons and axons from degeneration and to promote axonal regrowth.

Neuropeptide regulation of reactive gliosis

Reactive gliosis is a collective term that refers to the heterogeneous responses of astrocytes and microglia to acute and chronic brain injury, and is observable at some level in all neurodegenerative diseases. In acute injury, both microglia and astrocytes change morphology and release growth factors, cytokines, chemokines and other immunomodulators. Several of these alterations in glia cellular and molecular phenotype persist in chronic injury and neurodegenerative diseases. Although commonly viewed as harmful due to its association with proinflammatory molecules and scar tissue production, reactive gliosis has probably evolved as an important defensive reaction that attempts to minimize injury and promote repair. For example, several recent studies in genetically modified mice have indicated that interference with astrogliosis can worsen the course of CNS injury (Okada *et al.*, 2006; Herrmann *et al.*, 2008; Nobuta *et al.*, 2012), and it is clear that microgliosis plays a role in repair processes (reviewed in Carson *et al.*, 2006; Ransohoff, 2007).

Functional VIP and PACAP receptors are present on both astrocytes and microglia in culture, raising the possibility that VIP and PACAP provide some of their neuroprotective and regenerating actions via these glial cell types. In this regard, a number of studies have shown that VIP and PACAP can potential regulate the proliferation and morphology of astrocytes in culture and induce the release of cytokines and growth and survival factors (reviewed in Dejda *et al.*, 2005; Nakamachi *et al.*, 2011). *In vitro* and *in vivo* studies also support that VIP and PACAP can modulate microglia activity by altering the production of cytokines, chemokines and other molecules (Kim *et al.*, 2000; Wainwright *et al.*, 2008). The latter is not at all surprising give the abundant data indicating that these peptides are potent modulators of MΦ function (see below).

VIP and PACAP action on neural stem cell fate

Subsequent to CNS insults and other pathological conditions, neural stem cells, which are normally relatively quiescent, actively proliferate, migrate to lesion sites and differentiate in an attempt to salvage or repair damaged circuits. Several studies have examined the actions of VIP and PACAP on the fate of neural progenitors and stem cells *in vitro* (extensively reviewed in Nakamachi *et al.*, 2011). These have demonstrated diverse PAC_1, $VPAC_1$ and $VPAC_2$-mediated actions on proliferation, survival and neuronal and glial differentiation, and underscore the importance of the source and developmental stage of stem/progenitors, and the experimental conditions used for study. One recent investigation addressed receptor-mediated actions on neural stem cells *in vivo*,

showing that VPAC2-deficient mice exhibited a specific reduction in nestin-positive precursors in the dentate subgranular stem cell niche, as well as a reduction in the number of newly generated and surviving neurons in the dentate gyrus (Zaben *et al.*, 2009). So far, no studies have examined PACAP action on stem cell fate in neuroinflammatory and other forms of CNS injury or pathology.

The role of VIP and PACAP in the regulation of systemic inflammation

VIP immunoreactivity is present in the innervation of lymph nodes, thymus and other lymphoid tissue (Bellinger *et al.*, 1997), leading to the suggestion that neuronal sources of VIP might be used to regulate immune cell activity that is initiated, amplified or otherwise regulated in these lymphoid tissues. Interestingly, VIP and PACAP are also expressed in several subpopulations of immune cells (Gomariz *et al.*, 1993; Gaytan *et al.*, 1994; Leceta *et al.*, 1996; Abad *et al.*, 2002), providing potential non-neuronal sources of these peptides to regulate peripheral immune responses. The importance of an immune source of VIP during inflammation was suggested by the fact that a variety of inflammatory stimuli were shown to induce VIP production and release from cultures of lymph nodes, spleen and thymus (Martinez *et al.*, 1999), and from purified Th2-differentiated CD4+ Th lymphocytes (Vassiliou *et al.*, 2001). Finally, a bone marrow chimera approach was recently used to show that expression of VIP in radiosensitive haematopoietic cells was required to constrain Th polarization in a viral infection model (Li *et al.*, 2011). Although Th polarization occurs mainly if not exclusively in the periphery, the latter finding raises the general possibility that VIP (or PACAP) produced in immune cells, including microglia (Nishimoto *et al.*, 2011), are involved in modifying local inflammation or degeneration in the CNS

Nearly all immune cell types express one or more VIP and PACAP receptor subtypes. $VPAC_1$ and $VPAC_2$ receptors have been implicated in most of the immunomodulatory actions of PACAP and VIP (reviewed in Delgado *et al.*, 2004a), although PAC_1 receptors are expressed in MΦ, where they appear to have functional significance in innate (Martinez *et al.*, 2002), and perhaps adaptive immunity (Delgado *et al.*, 1999b,c). MΦ express constitutively $VPAC_1$ and PAC_1 receptors, and when exposed to inflammatory stimulus express $VPAC_2$ (Delgado *et al.*, 1999b). VIP and PACAP actions on various immune cell types in culture has undergone considerable investigation (extensively reviewed in Delgado *et al.*, 2004a; Yadav and Goetzl, 2008), and only certain critical points will be highlighted here. VIP and PACAP were repeatedly shown to inhibit the LPS-induced production of proinflammatory cytokines such as TNF-α, IL-6 and several chemokines in MΦ cultures, and to increase the synthesis and release of IL-10 and the IL-1 receptor antagonist (IL-1Ra; Delgado *et al.*, 1999a; Delgado and Ganea, 2001). VIP and PACAP also appear to play an important role in regulating T-cell biology. Both neuropeptides act on MΦ and dendritic cells to modify the expression of costimulatory molecules B7.1 and B7.2, with a resultant stimulatory activity on Th cells (Delgado *et al.*, 2000; 2004b). $CD4^+$ T-cells also express $VPAC_1$, and are induced to express $VPAC_2$ during differentiation to the Th2 phenotype. Evidence suggest that VIP can promote a positive Th2 to Th1 balance by acting directly and specifically on Th2-differentiated cells to increase their proliferation, survival and chemotaxis (reviewed in Delgado *et al.*, 2004a). More recent experiments indicate that exogenously administered VIP and PACAP may regulate the production or expansion of regulatory T-cells (Tregs) Fernandez-Martin *et al.*, 2006 and see below). Other well-described immunomodulatory actions mediated by VIP and PACAP receptors include regulation of chemotaxis and modulation of matrix metalloproteinases (reviewed in Delgado *et al.*, 2004a). The intracellular signalling mechanisms by which VIP and PACAP regulate various aspects of immune cell physiology are cell type and context dependent, and involves cAMP-dependent and cAMP-independent pathways, which impinge on the classical PKA, NF-kB and STAT signalling pathways (see Delgado *et al.*, 2004a for a more detailed discussion).

Dissection of VIP and PACAP actions in the experimental autoimmune encephalomyelitis (EAE) model of MS

Like that observed in other inflammatory diseases (Tornwall *et al.*, 1994; Belai *et al.*, 1997; Boyer *et al.*, 2007; Juarranz *et al.*, 2008), patients with MS have reported alterations in components of the VIP/PACAP signalling system. For example, patients with MS reportedly have decreased levels of VIP in their cerebral spinal fluid (Andersen *et al.*, 1984; Sharpless *et al.*, 1984), aberrant regulation of $VPAC_2$ receptors in lymphocytes when stimulated *in vitro*, and a distinct DNA footprinting pattern in the promoter region of the $VPAC_2$ gene (Sun *et al.*, 2006). These studies imply that VPAC receptor signalling may be altered either as a part of the pathology of MS or as an attempt to control inflammation. We and other have used the EAE model to study the potential significance of VIP and PACAP signalling in MS. In addition to being a widely used model for MS research, EAE provides an excellent experimental system to investigate specific interactions of the immune system with the brain as well as tolerance mechanisms that operate to prevent autoimmune diseases. This is possible because EAE can be reproducibly induced with defined CNS antigens, employs physiologically relevant processes that regulate flux of peripheral immune cells into the brain and involves well-characterized innate and adaptive immune mechanisms that promote inflammatory disease and normally prevent autoimmunity. Briefly, the classical EAE model is induced by immunization with a myelin peptide fragment such as $MOG_{35–55}$ (amino acids 35–55 of myelin oligodendrocyte glycoprotein). This results in the generation of autoreactive T-cells, which subsequently invade the CNS to initiate EAE, eventually culminating in myelin destruction.

The first use of this model to study the immunomodulatory actions of VIP or PACAP was reported almost 10 years ago (Kato *et al.*, 2004). In that study, PACAP was administrated i.p. to C57BL/6 mice every other day after immunization with $MOG_{35–55}$. PACAP administration was found to

ameliorate both the clinical and pathological manifestations of EAE. Splenocyte cultures from these mice exhibited significantly reduced MOG-specific IFN-γ production. In more extensive work by another group focusing on the therapeutic action of VIP, the clinical and pathological scores in chronic $MOG_{35–55}$-induced EAE in C57BL/6 mice were dramatically reduced by a 3 day VIP treatment either during the induction or after the onset of disease (Fernandez-Martin *et al.*, 2006; Gonzalez-Rey *et al.*, 2006). Three-day treatment with either VIP or PACAP was also found to ameliorate EAE in a relapsing-remitting model, with a blockade of symptoms lasting 60 days. This was associated with decreased spinal cord levels of several proinflammatory cytokines, chemokines and chemokine receptors, and increased levels of the anti-inflammatory cytokines IL-10, IL-1Ra and TGF-β, the latter occurring despite a fourfold reduction in the number of inflammatory cells in the spinal cord. Lymph nodes from VIP-treated mice showed reduced antigen-induced proliferation, and lower and higher productions of Th1 and Th2 cytokines respectively. Finally, in a relapsing-remitting EAE model, the administration of VIP resulted in the expansion of Tregs ($CD4^+CD25^+Foxp3^+$) in the periphery and the nervous system. These Tregs were reported to suppress T-cell activation to a greater extent than Tregs from untreated mice (Fernandez-Martin *et al.*, 2006).

These rather profound anti-inflammatory actions of administered VIP and PACAP in these models raised the question of whether or not the endogenously produced peptides played protective roles in inflammation. We thus characterized the EAE phenotype of PACAP- and VIP-deficient (KO) mice (C57BL/6) using the $MOG_{35–55}$ model (Tan *et al.*, 2009; Abad *et al.*, 2010). Clinical disease in PACAP KO mice was much more severe than in WT mice, involving four limb paralyses and requiring euthanization in 30% of cases. The increased sensitivity was accompanied by enhanced mRNA expression of Th1 and Th17 cytokines in the spinal cord, but down-regulation of Th2 cytokines. *Ex vivo* antigen-rechallenge assays indicated that PACAP KO mice exhibited increased T-cell proliferation in response to antigen, with a more pronounced induction of Th1 and Th17 cytokines. Moreover, the relative abundance of $CD4^+CD25^+FoxP3^+$ Tregs in lymph nodes and levels of FoxP3 mRNA in the spinal cord were reduced. In addition to demonstrating that endogenous PACAP protects against EAE by controlling cytokine responses, the results suggested that PACAP might function as one of the few known intrinsic regulators of Tregs. The type of Tregs affected in this model are natural Treg (nTregs), as opposed to inducible Tregs (iTregs, also called adaptive Tregs), which are mainly generated in models of more chronic inflammation. To this point, it has been clearly demonstrated in C57BL/6 mice that the induction of Tregs during $MOG_{35–55}$-induced EAE comes about exclusively by the expansion of the nTreg population, and not to the *de novo* production of iTregs from naïve T-cells (Korn *et al.*, 2007). Thus, the impairment in Treg expansion in PACAP KO mice in this model appears to involve primarily nTregs.

In contrast to the hyper-inflammatory phenotype of PACAP-deficient animals, VIP KO mice exhibited a paradoxical, nearly complete resistance to $MOG_{35–55}$-induced EAE (Abad *et al.*, 2010). The EAE resistance in VIP KO mice was reversed by pre-administering VIP for 2 weeks prior to EAE induction, demonstrating that this unexpected phenotype was indeed due to specific loss of VIP. Despite the absence of disease in VIP KO mice, immune cells isolated from the lymph nodes of VIP KO mice responded robustly to MOG *in vitro*, and induced EAE when transferred to WT recipient mice. Thus, VIP KO mice developed an aggressive T-cell response to MOG immunization, but clinical disease in these mice was blocked at a step downstream from immunization. In agreement, MOG-specific T-cells generated in wild-type mice could induce EAE when transferred into WT but not VIP KO recipients. Remarkably, inflammatory cells accumulated in the CNS in MOG-treated VIP KO mice, but seemed to be 'trapped' in the meningeal space and rarely invaded the parenchyma. This suggested that trans-migration of immune cells into the CNS parenchyma may be impaired in VIP KO mice, and demonstrated that VIP is somehow required for the EAE process to be fully manifested (Abad and Waschek, 2011). While the finding raises important questions for future study, the presence of this resistance has so far precluded an investigation in EAE of how endogenous VIP might modulate Treg production and other aspects of neuroinflammation.

Our more recent experiments have focused on mechanisms to explain the reduction of Treg abundance in PACAP KO mice. Initially, we focused on potentially defective mechanisms regulating the expansion of $FoxP3^+$ Tregs of PACAP KO mice by FACS using Ki67 as a mitotic marker, and Annexin V and 7-AAD to detect apoptotic cells. These studies, performed at the peak of disease (day 14) and during recovery (day 20) revealed that both proliferation and survival rates of $FoxP3^+$ Tregs in the lymph nodes were increased in WT mice during the course of EAE, but that these increases were markedly blunted in PACAP KO mice. In the CNS, a defect was observed in proliferation only. We also examined the proliferation rate of committed $FoxP3^+$ natural Tregs in their site of origin, the thymus, at the peak of disease. As in the lymph nodes and CNS, EAE enhanced the proliferation of $FoxP3^+$ Tregs in the thymus in WT mice, but this was significantly blunted in PACAP KO mice. These results revealed that PACAP is critically required to expand and maintain populations of committed $FoxP3^+$Tregs in the thymus as well as extrathymic sites.

A speculative model for VIP and PACAP involvement in the regulation of peripheral inflammation

The above analyses demonstrate that loss of VIP and PACAP critically affects important aspects of inflammation and tolerance during EAE, but provide little detail with respect to exactly where, when and how the endogenous neuropeptides act. In fact, relatively little is known with respect to how neurons in the CNS respond to and control local CNS inflammation. On the other hand, considerable mechanistic information is available to explain how the brain controls systemic inflammation by way of innervation of lymph nodes, spleen, thymus and bone marrow, where the activation and expansion of immune cells to a large extent occurs. Much has come from investigation of the so-called inflammatory reflex, in which inflammation in the periphery is

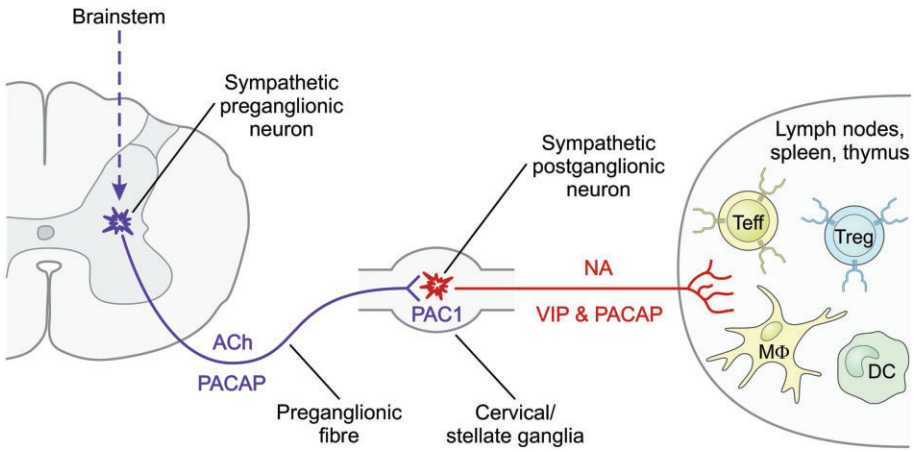

Figure 2

Potential neural circuitry by which PACAP and VIP modulate peripheral immune cell activity during inflammatory stress. Neurons in the brainstem and hypothalamus (not shown) sense inflammation and activate preganglionic sympathetic neurons in the spinal cord IML column. PACAP is expressed with ACh in the preganglionic neurons in thoracic spinal cord IML neurons. When released during stress or inflammation, PACAP acts via PAC_1 receptors expressed on sympathetic neurons in cervical/stellate ganglia to alter NA synthesis and/or activity, and to increase the production of VIP or PACAP. The latter neuropeptides are released along with NA in peripheral immune sites such as the thymus, lymph nodes and spleen. VIP or PACAP may then act directly with $VPAC_1$, $VPAC_2$ or PAC_1 receptors on immune cells, or indirectly via action on stromal cells expressing these receptors. Teff, T effector cell (Th1, Th2 and Th17); DC, dendritic cells.

sensed in the brain stem and hypothalamus, then transmitted to the periphery via the autonomic nervous system, in particular the sympathetic nervous system (SNS; Figure 2). Historically, a neural circuit coined 'the cholinergic anti-inflammatory pathway', mediated prototypically by the vagus nerve, was shown to have important roles in regulating the immune response in several experimental disease models, including sepsis, haemorrhagic shock, pancreatitis and post-operative ileus (reviewed in Rosas-Ballina and Tracey, 2009; Tracey, 2009; Thayer and Sternberg, 2010). However, despite an abundance of supporting functional data, the concept of this circuit was considered highly controversial because few investigators could provide solid evidence for cholinergic innervation of the spleen, lymph nodes or other immune organs. The conundrum seems to have been resolved to some extent in recent years with data supporting a model whereby vagal stimulation of the celiac or other sympathetic ganglia results in release of noradrenaline (NA) in lymphoid organs such as the spleen. NA then acts on MΦ in these organs to release ACh, which in turn acts in an autocrine or paracrine manner on immune cells that express α7 nicotinic receptors to alter their function (Rosas-Ballina and Tracey, 2009; Tracey, 2009; Thayer and Sternberg, 2010). When viewed separately, the concept of SNS control of immune responses, although still controversial, appears more straightforward (reviewed in Nance and Sanders, 2007; Bellinger *et al.*, 2008). For example, it is well documented that peripheral lymphoid organs, including spleen, lymph nodes, thymus and probably bone marrow, receive abundant innervation from sympathetic ganglia, with nerve terminals residing adjacent to immune and stromal cells.

The role of the SNS in controlling inflammation has been examined primarily by peripheral administration of 6-hydoxydopamine, which does not appreciably penetrate the blood–brain barrier, and thus selectively ablates sympathetic neurons in the periphery. Most of these studies, as well as studies in which immune cells were treated with NA receptor analogues *in vitro*, suggested that NA acts primarily via β-2 adrenoreceptors, and mainly inhibits innate inflammatory responses, and either promotes or inhibits adaptive immunity (Nance and Sanders, 2007; Bellinger *et al.*, 2008). Unfortunately, very limited illuminating data are available so far which address this problem at the molecular level using genetically engineered mice. For example, a comprehensive analysis of the immune phenotype of β-2 adrenoceptor-deficient mice reported essentially normal immune responses (Sanders *et al.*, 2003). On the other hand, dopamine β-hydroxylase-deficient mice were found to exhibit diminished Th1 responses to pathogen challenge (Sanders *et al.*, 2003), providing evidence that SNS actions might subserve, rather than inhibit, inflammation in this context. Overall, the studies using sympathectomy, genetically engineered mice, and *in vitro* assays have generally not examined the role of other signalling molecules, such as neuropeptides, that are released by sympathetic neurons or their presynaptic innervation.

VIP and PACAP are expressed in this circuitry, and are known to regulate sympathetic function in other contexts. In this regard, PACAP is expressed in the Ach-expressing preganglionic neurons of the sympathetic ganglia, including those that innervate the thymus and cervical lymph nodes (Beaudet *et al.*, 1998; Pettersson *et al.*, 2004; Figure 2). Retrograde tracing with pseudorabies virus and other anatomical methods demonstrate that neurons in the superior cervical ganglia (SCG), which innervate the thymus and cervical lymph nodes, receive their presynaptic innervation from the thoracic intermediolateral (IML) neurons in the spinal cord. At least two groups have shown that IML neurons abundantly express PACAP and PACAP mRNA (Beaudet *et al.*, 1998; Pettersson *et al.*, 2004), and furthermore, more than half of

the IML preganglionic neurons that project to the SCG express PACAP gene transcripts (Beaudet *et al.*, 1998). PACAP and PACAP gene transcripts are also expressed in locations of higher order neurons that regulate sympathetic outflow to the lymphoid organs. There is good evidence that PACAP generally functions in sympathetic neurons to alter tone and/or activity. For example, PACAP has been shown to increase electrical activity in sympathetic nerves in anaesthetized rats (Tanida *et al.*, 2010), and to induce catecholamine release and tyrosine hydroxylase gene expression in cultured SCG (May and Braas, 1995). Moreover, PACAP KO mice exhibit a thermogenesis defect in the first 2 weeks of life that is linked to a deficit in brown fat metabolism, a process regulated by sympathetic tone (Gray *et al.*, 2002). In this respect, levels of NA and two enzymes involved in fat metabolism regulated by NA were significantly reduced in these mice. In a metabolic stress model (insulin challenge), hypoglycaemia was more profound and longer lasting in PACAP KO than WT mice, and was associated with impaired long-term secretion of epinephrine, a lack of induction of tyrosine hydroxylase activity in the adrenal medulla and a depletion of adrenomedullary epinephrine stores. Finally, an additional mode of PACAP action is suggested by the fact that PACAP induces its own gene expression as well as that of VIP in cultured sympathetic neurons (Braas *et al.*, 2007) via action on PACAP-selective PAC_1 receptors (the only PACAP receptor on sympathetic neurons; Girard *et al.*, 2004; Braas *et al.*, 2007). Accordingly, PACAP from preganglionic neurons might trigger the release of VIP or PACAP in postsynaptic neurons, which then may act directly or indirectly on immune cells within peripheral lymphoid organs such as the thymus and lymph nodes. The fact that most immune cells express VIP and PACAP receptors suggests that postsynaptic release of VIP and/or PACAP in the target organ might mediate the effects of presynaptic PACAP independent of its actions on NA release and/or activity. Alternatively, as previously discussed, PACAP might be available from other relevant sources such as T lymphocytes (Gaytan *et al.*, 1994; Abad *et al.*, 2002). Future study employing cell-specific gene targeting and other approaches will be necessary to test various aspects of this model.

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Conflicts of interest

None declared.

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